The hemagglutinin protein of influenza A/Vietnam/1203/2004 (H5N1) contributes to hyperinduction of proinflammatory cytokines in human epithelial cells

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ABSTRACT

Live attenuated influenza A/Vietnam/1203/04 (H5N1) (VN04 cold adapted [ca]) and A/Hong Kong/213/03 (H5N1) (HK03 ca) vaccine viruses were compared with the A/New Caledonia/20/99 (H1N1) (NC99 ca) seasonal vaccine virus for induction of host gene expression in infected human epithelial cells. Levels of proinflammatory cytokines and interferon-related genes were significantly upregulated in VN04 ca virus-infected A549 cells compared to cells infected with the HK03 ca and NC99 ca viruses as examined by microarray analysis and confirmed by quantitative RT-PCR and ELISA assays. Further mapping studies demonstrated that the hemagglutinin (HA) protein of the VN04 ca virus contributed to the hyperinduction of cytokines. The inactivated viruses could also induce the production of the cytokines and chemokines, albeit at a much lower level than live viruses. Compared to HK03 ca virus, VN04 ca virus differs by 9 amino acids including an additional glycosylation site at residue 158N of the HA protein and a shortened stalk in the neuraminidase (NA) protein. Increased cytokine production by HK03 ca virus was only observed when HK03 ca virus acquired an additional glycosylation in the HA protein and when its NA protein was replaced by that of VN04. Thus, our data indicate that the HA protein and its interaction with the NA protein play a role in triggering cytokine responses. The full implications of cytokine induction in vaccine virus-induced immune responses remain to be explored.

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Introduction

Human infection with the highly pathogenic avian influenza (HPAI) H5N1 viruses was first reported in 1997 (Claas et al., 1998), and since 2003, more than 400 cases have been diagnosed with a mortality rate as high as 60% (WHO, Global Alert and Response program (GAR); http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_12_30/en/index.html). Although H5N1 infections have primarily been caused by avian-to-human transmission, the widespread distribution of H5N1 viruses among birds and domestic poultry provides an opportunity for the HPAI H5N1 viruses to adapt to the human host and/or to reassort with human influenza viruses; this may potentially allow an H5N1 avian virus to become more efficient in human-to-human transmission and to cause an influenza pandemic.

Human infection with HPAI H5N1 viruses is characterized by a rapid, progressive viral pneumonia associated with leucopenia, serum cytokine elevation, and multiorgan failure (Abdel-Ghafar et al., 2008; Yuen et al., 1998). Systemic spread of HPAI H5N1 viruses to multiple organs (de Jong et al., 2005). The multibasic amino acid motif in the hemagglutinin cleavage site (Hatta et al., 2001; Steinhauer, 1999) and the dysregulation of cytokine and chemokine expression are thought to contribute at least partially to systemic tissue damage and disease severity (de Jong et al., 2006; Peiris et al., 2004). HPAI H5N1 viruses appear to be different from seasonal influenza viruses in triggering the induction of cytokines and chemokines in human infections (de Jong et al., 2006; Peiris et al., 2004; To et al., 2001) as demonstrated in infected human monocyte-derived macrophages (Cheung et al., 2002; Guan et al., 2004; Mok et al., 2007), in respiratory epithelial cells (Chan et al., 2005), and in infected mice and ferrets (Cameron et al., 2008; Perrone et al., 2008; Szretter et al., 2007; Tumpey et al., 2000).

Toward the goal of preventing or controlling influenza pandemics caused by the HPAI H5N1 viruses, we have developed several live attenuated pandemic H5N1 influenza vaccines (pLAI). These vaccines contain: (1) the cleavage site-modified hemagglutinin (HA) gene; (2) the neuraminidase (NA) gene segments from the H5N1 viruses A/Vietnam/1203/04 (VN04), A/Hong Kong/491/97 (HK97) or A/Hong Kong/213/03 (HK03); and (3) the six internal protein gene segments (PB1, PB2, PA, NP, M, and NS) from cold adapted (ca) A/Ann Arbor/6/60 (H2N2, AA60 ca), the same master donor virus that is used for the influenza A vaccine components of the commercial seasonal LAIV. Although each of the three H5N1 vaccine strains provided complete protection against lethal challenge with a wild-type (wt) H5N1 virus in mice and prevented pulmonary replication of wt virus in ferrets (Suguitan et al., 2006), these live virus vaccines were highly restricted in replication in healthy adults.
genes have previously been reported to be highly expressed in H5N1 VN04 wt virus-infected ferrets (Cameron et al., 2008), cynomolgus macaques (Baskin et al., 2009), and humans (de Jong et al., 2006). Higher levels of type I IFN-related genes, including the components and induced low humoral antibody responses in phase 1 clinical studies (Karron et al., 2009). Previously, we observed that intranasal administration of H5N1 VN04 ca virus, at a high dose and in large volume, caused mild to moderate bronchopulmonary inflammation in ferrets compared to minimal pathological changes in the lungs of ferrets infected with the H5N1 HK03 ca or H1N1 A/New Caledonia/20/1999 (NC99) ca viruses (Jin et al., 2007). Unlike the wt influenza viruses used in the reported studies, the H5N1 and H1N1 ca viruses differ only in the HA and NA proteins, suggesting that the glycoproteins of the VN04 ca virus may be involved in the increased induction of the proinflammatory immune response.

The H5N1 VN04 ca virus has receptor binding preference for α2,3-linked sialosides (α2,3SAL) and is poorly immunogenic in mice and ferrets. In contrast, the HK03 ca virus binds to both α2,3SAL and α2,6SAL and is more immunogenic than VN04 ca in animals. We have recently reported that α2,3SAL receptor binding specificity and 158N glycosylation contributed to the ability of the VN04 ca virus to replicate efficiently in the upper airway and to induce humoral antibody responses in ferrets (Wang et al., 2010). To understand whether differential inflammatory responses in vaccine virus-infected ferrets might be associated with virus immunogenicity, we examined host gene expression in human epithelial cells infected with the H5N1 VN04 ca, HK03 ca, and NC99 ca viruses by cDNA microarray analysis. We found that a panel of proinflammatory cytokines and chemokines were significantly upregulated in VN04 ca virus-infected cells compared to those cells infected with the HK03 ca or NC99 ca virus, although the viruses replicated to similar levels. Using reassortant ca viruses containing the HA and NA genes from VN04, HK03, or NC99, we found that the HA protein of the VN04 ca virus contributed to the hyperinduction of cytokine responses.

**Results**

**Differential gene expression in virus-infected cells**

To provide a global view of the host gene expression in response to infection of the H5N1 ca viruses in comparison to that of the H1N1 ca virus in human epithelial cells, cDNA microarray analysis of RNAs isolated from uninfected A549 cells and cells infected with the H5N1 VN04 ca, HK03 ca, or H1N1 NC99 ca viruses was performed. To ensure that the three viruses had similar replication kinetics in A549 cells, levels of viruses released into the culture supernatants at 0, 6, 24, and 48 hr PI were examined (Fig. 1). The HK03 ca virus replicated to a titer that was slightly higher than that of VN04 ca and NC99 ca virus infections throughout the 48-hr growth period. The numbers of cells that were infected with each virus at 6 hr PI were similar as assessed by immunofluorescence staining. Western blotting analysis showed that the NP proteins expressed in these virus-infected cells at 6 hr PI were comparable.

Equal quantities of total RNA from virus-infected or mock-infected A549 cells were processed for gene expression analysis at 6 hr PI. Virus infection was conducted in triplicate and the genes that had expression levels of 2-fold higher, or lower, than mock-infected cells were filtered using the cutoff value of 2.0. A total of 627, 894, and 650 genes were found to be up- or downregulated in response to VN04 ca, NC99 ca, or HK03 ca virus infection, respectively (Fig. 2). Further analysis indicated that the genes induced by VN04 ca were expressed at levels significantly higher than by NC99 ca or HK03 ca virus infection (Fig. 2, Venn diagrams). Table 1 lists a total of 31 cytokine-related genes (proinflammatory, chemotactic, and antiviral) in VN04 ca virus-infected cells that were induced to a level ≥1.5-fold higher than observed in NC99 ca virus-infected cells. Most of these genes are related to inflammatory and innate immunity, such as the cytokines, chemokines, and IFN-related genes, IFN response, and regulation. CXCL10, CCL2 (MCP-1), CCL5 (RANTES), TNF-α, and IFN-β were significantly upregulated in VN04 ca virus-infected cells, and these

**Fig. 1.** Replication of the H1N1 ca or H5N1 ca virus in A549 cells. A549 cells were infected with each virus at an MOI of 2.0. One set of the cell monolayers was fixed at 6 hr PI and stained with anti-influenza A NP antibody, and the other set was used for Western blotting by anti-AA ca antibody to detect the NP protein. Aliquots of culture supernatants from a replicate infection were taken at 6, 24, and 48 hr PI for virus titration in MDCK cells by plaque assay, and virus titers are represented in the growth curves.

<table>
<thead>
<tr>
<th>Regulated Gene</th>
<th>NC99</th>
<th>VN04</th>
<th>HK03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td>160</td>
<td>269</td>
<td>171</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>467</td>
<td>625</td>
<td>479</td>
</tr>
<tr>
<td>Total</td>
<td>627</td>
<td>894</td>
<td>650</td>
</tr>
</tbody>
</table>

**Fig. 2.** Differentially expressed genes in infected A549 cells by microarray analysis. A549 cells were mock infected or infected with the NC99 ca, VN04 ca, or HK03 ca viruses at an MOI of 2.0. At 6 hr PI, total cellular RNA was extracted for microarray analyses. A number of significantly differentially expressed genes as defined by >2-fold change (P < 0.05) using unpaired Student’s t-test were identified in the infected A549 cells compared to the mock infected cells. The data represent the results from three independent infections for each virus. The total numbers of differentially expressed genes in NC99, VN04, or HK03 ca virus-infected A549 cells are tabulated, and the numbers of genes specific to individual virus or shared by the vaccine viruses are represented in the Venn diagram.
Proinflammatory and chemotactic genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change over mock</th>
<th>Fold change over NC99</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>Interleukin 1, alpha</td>
<td>1.9</td>
<td>4.7</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
<td>5.4</td>
<td>11.0</td>
</tr>
<tr>
<td>IL-28</td>
<td>Interleukin 28A (IFN, lambda 2)</td>
<td>474.7</td>
<td>835.4</td>
</tr>
<tr>
<td>IL-28A/28B</td>
<td>Interleukin 28A/28B</td>
<td>138.0</td>
<td>343.3</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>3.5</td>
<td>8.9</td>
</tr>
<tr>
<td>CXCL10 (IP10)</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>59.9</td>
<td>128.2</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Chemokine (C-X-C motif) ligand 11</td>
<td>40.8</td>
<td>119.2</td>
</tr>
<tr>
<td>CCL4 (MIP-1α)</td>
<td>Chemokine (C-C motif) ligand 4</td>
<td>1.2</td>
<td>4.9</td>
</tr>
<tr>
<td>CCL5 (RANTES)</td>
<td>Chemokine (C-C motif) ligand 5</td>
<td>37.4</td>
<td>117.8</td>
</tr>
<tr>
<td>CCL2 (MCP-1)</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>7.0</td>
<td>11.7</td>
</tr>
<tr>
<td>CCL20</td>
<td>Chemokine (C-C motif) ligand 20</td>
<td>2.2</td>
<td>8.0</td>
</tr>
<tr>
<td>PLAUR</td>
<td>Plasminogen activator, urokinase receptor</td>
<td>3.6</td>
<td>7.0</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>Tumor necrosis factor, alpha-induced protein 3</td>
<td>2.8</td>
<td>6.1</td>
</tr>
</tbody>
</table>

IFN-related genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change over mock</th>
<th>Fold change over NC99</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFIT1</td>
<td>IFN-induced protein with tetratricopeptide repeats 1</td>
<td>442.0</td>
<td>658.2</td>
</tr>
<tr>
<td>IFIT3</td>
<td>IFN-induced protein with tetratricopeptide repeats 2</td>
<td>160.3</td>
<td>301.6</td>
</tr>
<tr>
<td>IFIT2</td>
<td>IFN-induced protein with tetratricopeptide repeats 2</td>
<td>83.1</td>
<td>168.9</td>
</tr>
<tr>
<td>ISG20</td>
<td>IFN stimulated exonuclease gene 20 kDa</td>
<td>4.6</td>
<td>8.7</td>
</tr>
<tr>
<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>113.4</td>
<td>282.2</td>
</tr>
<tr>
<td>IFIH1</td>
<td>IFN induced with helicase C domain 1</td>
<td>32.3</td>
<td>66.7</td>
</tr>
<tr>
<td>IFTN1</td>
<td>IFN, beta 1, fibrolast</td>
<td>20.0</td>
<td>59.8</td>
</tr>
<tr>
<td>IL-29</td>
<td>Interleukin 29 (IFN, lambda 1)</td>
<td>38.5</td>
<td>120.4</td>
</tr>
<tr>
<td>IRF1</td>
<td>IFN regulatory factor 1</td>
<td>29.6</td>
<td>43.8</td>
</tr>
<tr>
<td>DDX58</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</td>
<td>15.7</td>
<td>32.7</td>
</tr>
<tr>
<td>MDX1</td>
<td>MAX dimerization protein</td>
<td>8.1</td>
<td>16.6</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
<td>8.0</td>
<td>15.4</td>
</tr>
<tr>
<td>MX1</td>
<td>Myxovirus (influenza virus) resistance</td>
<td>7.6</td>
<td>11.2</td>
</tr>
<tr>
<td>RASD2</td>
<td>Radical S-adenosyl methionine domain containing 2</td>
<td>3.9</td>
<td>11.1</td>
</tr>
<tr>
<td>IFI44</td>
<td>IFN-induced protein 44</td>
<td>1.6</td>
<td>6.3</td>
</tr>
<tr>
<td>OASL</td>
<td>2′-5′-oligoadenylate synthetase-like</td>
<td>76.4</td>
<td>190.1</td>
</tr>
<tr>
<td>PTX3</td>
<td>Pentraxin-related gene, rapidly induced by IL-1 beta</td>
<td>1.8</td>
<td>5.3</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>3.2</td>
<td>5.2</td>
</tr>
</tbody>
</table>

* Mean gene expression is normalized to the corresponding mock infection. Significant change in gene expression versus mock infection is defined as P ≤ 0.05 by Student’s t tests.

Differentially expressed cytokine-related genes in H5N1 ca and H1N1 ca virus-infected cells.

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change over mock</th>
<th>Fold change over NC99</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>VN04 HK03 VN04 HK03</td>
<td>VN04 HK03 VN04 HK03</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.8 2.4 1.6 1.2</td>
<td>3.9 2.5 1.8 0.9</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.5 2.1 1.8 1.4</td>
<td>3.5 2.0 1.6 0.8</td>
</tr>
<tr>
<td>CCL2</td>
<td>2.3 1.9 1.6 1.2</td>
<td>3.0 2.5 1.9 1.0</td>
</tr>
</tbody>
</table>

Microarray analysis showed that a number of genes involved in the type I IFN activation pathway were differentially upregulated in H5N1 VN04 ca virus-infected cells (Table 1). qRT–PCR analysis performed to examine the expression levels of several IFN-related genes confirmed that RIG-I and MX-1 mRNAs were upregulated in virus-infected cells (Fig. 3A; data not shown). VN04 ca virus-infected cells had higher levels of RIG-I and IFN-α gene expression compared to cells infected with the H503 ca or NC99 ca virus. To further examine if the type I IFN gene activation pathway was differentially activated in virus-infected cells, IFN-β promoter activation was examined by transfection of a plasmid encoding a reporter gene under the control of the IFN-β promoter, followed by virus infection. As shown in Fig. 4B.

Differentially expressed genes analyzed by qRT–PCR were shown to be upregulated in H5N1 VN04 ca virus-infected cells compared to H1N1 NC99 ca virus-infected cells. Higher levels of IL-6 mRNA were detected in VN04 ca virus-infected cells than in those cells infected with the H503 ca or NC99 ca virus. The virus containing the HA from VN04 induced the highest levels of proinflammatory cytokines, including IL-8 and CCL5, indicating that the HA of VN04 played a major role in cytokine induction. The virus containing the HA from VN04 induced the highest levels of proinflammatory cytokines, indicating that the HA of VN04 played a major role in cytokine induction.

**Differential type I IFN response in virus-infected cells**

Microarray analysis showed that a number of genes involved in the type I IFN activation pathway were differentially upregulated in H5N1 VN04 ca virus-infected cells (Table 1). qRT–PCR analysis performed to examine the expression levels of several IFN-related genes confirmed that RIG-I and MX-1 mRNAs were upregulated in virus-infected cells (Fig. 3A; data not shown). VN04 ca virus-infected cells had higher levels of RIG-I and IFN-α gene expression compared to cells infected with the H503 ca or NC99 ca virus. To further examine if the type I IFN gene activation pathway was differentially activated in virus-infected cells, IFN-β promoter activation was examined by transfection of a plasmid encoding a reporter gene under the control of the IFN-β promoter, followed by virus infection. As shown in Fig. 4B.
IFN-β promoter activity was significantly stimulated in VN04, HK03, or NC99 virus-infected cells relative to uninfected cells; VN04 virus induced at least 4-fold higher activity than the NC99 virus and ~2-fold higher than the HK03 virus. As a result of the IFN-β promoter activation, a number of IFN-responsive or -inducible genes were also significantly upregulated in virus-infected cells as identified by the microarray analysis (Table 1) and by qRT-PCR that confirmed the up-regulation of ISG15 (Fig. 4B, right panel). The VN04 virus induced levels of ISG15 approximately 2-fold higher than those induced by the NC99 or HK03 virus. Thus,

Fig. 3. Cytokine gene expression in infected A549 cells. A549 cells were infected with the NC99, VN04, or HK03 virus at an MOI of 2.0 or were mock infected. (A) IL-6 gene expression by qRT-PCR and ELISA. IL-6 mRNA levels examined at 6 hr PI are presented as the relative fold-increase compared to mock-infected cells, and the values represent three independent infections. The secretion of IL-6 protein in the supernatant was analyzed by ELISA at 6 and 24 hr PI. IL-8, CCL5, CCL2, and CXCL10 production at 6, 24, or 48 hr PI were examined by ELISA. (B) The effect of HA protein on cytokine induction. A549 cells were infected with reassortant 7:1 virus at MOI 2.0. The secreted IL-8 and CCL5 proteins in the supernatants were examined at 6 and 24 hr PI by ELISA. The error bars represent variation from duplicate cultures. The dotted line indicates the level of the protein detected in mock infected cells. *P < 0.05, indicates the value of the VN04 or VN04-HA-containing virus infection that is statistically different from that caused by infections with NC99 or HK03 (A) or the viruses containing the NC99-HA and HK03-HA (B).
these data indicate that, relative to the HK03 ca and NC99 ca viruses, the VN04 ca virus was the most potent inducer. The HK03 ca virus appeared to be slightly more potent than the NC99 ca virus in IFN induction.

**Contributions of the HA and NA proteins to hyperinduction of cytokines**

We showed earlier that the N2 NA from AA ca virus did not affect the HA-induced host gene expression. To determine if the N1 NA protein contributed to virus-mediated cytokine and chemokine gene expression, reassortant ca viruses containing the HA and NA from the VN04, HK03, or NC99 ca viruses were generated and examined for the induction of cytokines in A549 cells (Fig. 5A). All viruses replicated to similar titers and expressed similar levels of viral proteins as shown by Western blotting (Fig. 5B). Interestingly, only the HK03 HA (CHO-158+) together with the HK03 NA or VN04 NA. All of the reassortants replicated similarly in infected A549 cells (data not shown) and expressed similar levels of NP proteins as shown by Western blotting (Fig. 5B). Interestingly, only the HK03 ca virus containing the additional glycosylation site (CHO-158+) paired with the VN04 NA caused higher cytokine induction, although the level of IL-8 was lower than that induced by the VN04 ca virus by approximately 3-fold. To examine if adding this 158N glycosylation site to HK03 ca virus would result in increased cytokine expression, mutagenesis was performed in the HK03 HA to introduce the glycosylation at 158N and virus was rescued by transfecting the HK03 HA (CHO-158+) together with the HK03 NA or VN04 NA. All of the reassortants replicated similarly in infected A549 cells (data not shown) and expressed similar levels of NP proteins as shown by Western blotting (Fig. 5B). Interestingly, only the HK03 ca virus containing the additional glycosylation site (CHO-158+) paired with the VN04 NA caused higher cytokine induction, although the level of IL-8 was lower than that induced by the VN04 ca virus. This result indicates that, in addition to the 158N glycosylation, the interaction between the HA and NA is also important in the hyperinduction of cytokines in VN04 ca virus-infected cells.

**Inactivated virus can also induce differential cytokine expression**

To determine whether virus-mediated cytokine induction was replication dependent or not, levels of cytokine induction in live virus-infected cells were compared to cells infected with BPL-inactivated virus. Virus was shown to be completely inactivated by BPL by plaque assay. As shown in Fig. 6A, the inactivated virus was able to stimulate the production of the CCL5 chemokine at 6 and 24 hr PI, albeit at levels

![Fig. 4. Activation of the IFN-β pathway in infected A549 cells. A549 cells were infected with NC99, VN04, or HK03 ca viruses at an MOI of 2.0 or were mock infected. Levels of IFN-β, IFN-α, and ISG-15 mRNA expression were analyzed by qRT-PCR. The fold changes of mRNA relative to mock-infected cells from three independent infections are presented. IFN-β promoter activity was examined by FF-Luc protein expression that is under the control of the IFN-β promoter together with the cotransfection of a control plasmid encoding a Ren-Luc gene. The transfected cells were superinfected with each virus at an MOI of 2.0 at 16 hr PI for 16 hr. The FF-Luc activity was normalized with the Ren-Luc activity and expressed as the relative light units compared to uninfected cells. The error bars represent variations between the two infections. *Value in VN04 ca virus-infected cells, which was statistically different from HK03 and NC99 ca virus infections (P < 0.05); †value in the HK03 ca virus-infected cells, which was statistically different from the NC99 ca virus infection (P < 0.05).](image)
of >2-fold lower than the live viruses. The inactivated VN04 ca virus remained the most potent cytokine inducer among the three inactivated viruses. Similar patterns were also observed for the induction of the IL-6 and IL-8 proteins (Fig. 6B). Thus, virus replication is not an absolute requirement for cytokine induction.

Discussion

In this study, we examined host gene expression profiles of a human respiratory epithelial cell line infected with either H5N1 ca or H1N1 ca viruses. A total of more than 1000 genes were found to be either up- or downregulated in A549 cells infected with the H5N1 VN04 ca, HK03 ca, or H1N1 NC99 ca virus. H5N1 VN04 ca virus infection caused more differential gene expression than the HK03 ca or NC99 ca viruses. Most significantly, upregulated genes in VN04 ca virus-infected cells were those that are involved in inflammation and innate immunity. These three ca viruses share six internal protein gene segments (PB1, PB2, PA, NP, M, and NS) from ca A/Ann Arbor/6/60, the differences among these viruses reside in the HA and NA proteins. Using reassortant viruses that contain the HA protein from three different viruses and the same N2 NA from A/Ann Arbor/6/60 ca virus or the N1 NA from other two viruses, we showed that the VN04 HA protein plays a major role in triggering hyperinduction of cytokines in virus-infected cells.

The NA protein has been previously found to regulate apoptosis by activating latent TGF-β (Morris et al., 1999; Schultz-Cherry and Hinshaw, 1996). Additional glycosylation on the HA globular head and a shortened NA stalk are characteristic features of the H5 and H7 chicken viruses (Matrosovich et al., 1999). A deletion in the NA stalk decreases its ability to release the virus from cells; the acquisition of a carbohydrate at the HA head compensates the low NA activity by reducing HA binding to the cell receptors. The stalk length and additional glycosylation of the HA have been shown to affect virulence of H5N1 viruses in mice (Matsuoka et al., 2009; Yen et al., 2009). Interestingly, when the additional glycosylation site was added to HK03 ca virus, it did not result in increased cytokine induction. Increased cytokine gene expression induced by HK03 that acquired 158N glycosylation was only observed when its NA gene was replaced by the VN04 NA gene that encodes a protein with a shorter stalk.
Removal of 158N glycosylation from VN04 virus resulted in reduced gene expression, but the reduced gene expression was not observed when the S223N change was also introduced into the VN04 HA that lacked 158N glycosylation (data not shown). The reduction in cytokine gene expression resulting from infection of the VN04-HA reassortant viruses (with the NA protein from different viruses) could be due to changes in the functional balance between the HA and NA proteins or to an NA-mediated differential response. Nevertheless, the reassortant viruses with the HA protein from VN04 induced higher production of cytokines than those with the HA protein from either the HK03 or NC99 ca virus.

Although the multibasic amino acid sequence was removed from the cleavage site of the HA of the VN04 ca virus, the VN04 ca virus retained the capacity to induce differential gene expression as was described for the VN04 wt virus (Cameron et al., 2008; Chan et al., 2005). The data obtained from the infected A549 cells are in agreement to what have been previously reported in a variety of hosts and cells infected with the H5N1 virus; these include (1) a significant increase of proinflammatory cytokines such as CXCL10, CCL5, and IL-6 induced by influenza A H5N1 wt viruses in primary human alveolar and bronchial epithelial cells (Chan et al., 2005); (2) the elevation of serum CXCL10 in humans with severe H5N1 infection (Kandun et al., 2006; Peiris et al., 2004); and (3) robust CXCL10 gene expression in VN04 wt virus-infected ferret lung tissues compared to those infected with A/Panama/2007/99 (H3N2) and significantly reduced disease severity and mortality by blocking the IP10 receptor CXCR3 with its antagonist (Cameron et al., 2008). IL-6 is considered to be an indicator of severe disease in humans (Kaiser et al., 2001); IL-6 induction correlates with severe seasonal influenza infection in ferrets (Svitek et al., 2008). We also observed higher levels of CXCL10, CCL5, CCL2, GM-CSF, and TNFα in VN04 ca virus-infected primary normal human infected bronchial epithelial cells (NHBE) overexpression levels in cells infected with the NC99 ca virus (data not shown). Thus, the hyperinduction of these cytokines is not limited to lung epithelial cells. The lung histopathology observed in VN04 ca virus-infected ferrets when the virus inoculum reached the lungs at a high dose and high volume (Jin et al., 2007) was likely due to the cytokine-hyperinducing nature of the VN04 ca virus.

How the virus triggers the innate immune response is not entirely known. Several viral glycoproteins have been reported to play a role in triggering the expression of proinflammatory cytokines and chemokines through Toll-like receptors (TLR), such as the F protein of respiratory syncytial virus (RSV) via TLR4 (Kurt-Jones et al., 2000), the HN protein of measles virus via TLR2 (Bieback et al., 2002), and the gB and gH proteins of cytomegalovirus via TLR2 (Compton et al., 2003). TLR7 and TLR8 have been shown to be involved in intracellular sensing of single-stranded RNA of live influenza virus by plasmacytoid dendritic cells (Diebold et al., 2004). Studies of the H1N1 reassortant viruses indicated that the HA protein of the pandemic 1918 virus appears to increase the virulence of the VN04-HA plasmid was performed. Infection of primary murine B lymphocytes via a MyD88-dependent pathway distinct from that involved in sensing viral RNA (Marshall-Claude et al., 2006). It was thus suggested that the activation of the MyD88-dependent pathway might be mediated by a novel member of TLR family or a novel MyD88-dependent and TLR-independent pathway. As in the case of the influenza H2 HA protein and the inactivated CVV that induced NF-κB activation and cytokine production in normal human dermal fibroblasts (Bieback et al., 2002), we also demonstrated that viral replication is not absolutely required for cytokine induction. Similar results were observed by examining the live and inactivated VN04 HA variant lacking glycosylation at residue 158N, indicating that the glycosylation site in the globular head of the VN04 HA may influence the interaction of the HA protein with an unknown factor(s).

Influenza virus infection results in the production of a variety of cytokines with chemotactic (CCL5, CCL2, CCL4, and CXCL10), proinflammatory (IL-1β, IL-6, IL-18, and TNF-α), and antiviral (IFN-α/β) properties (Julkunen et al., 2001). The chemokines produced in influenza virus infection preferentially favor the recruitment of mononuclear cells to the site of infection. Type I IFNs are the key cytokines produced by influenza virus-infected epithelial cells and monocytes/macrophages (Ronni et al., 1997; Ronni et al., 1995; Sareneva et al., 1998). Innate immunity is believed to be important in restricting the replication of influenza A virus at early stages of infection and in establishing the adaptive immunity that is required for viral clearance. It is postulated that cytokine-induced inflammatory responses play a significant role in acute respiratory distress syndrome (Peiris et al., 2009). Although the highly pathogenic H5N1 VN04 virus induced high levels of IFN gene expression in human and ferret infections, virus replication remained poorly controlled. Thus, it remains to be determined whether cytokine levels correlate with viral load in the nasopharynx and reflect increased virus replication or pathogenesis (de Jong et al., 2006; Deng et al., 2008). Despite higher levels of cytokines and chemokines induced by the VN04 ca virus in the infected A549 cells, replication of VN04 ca virus was not reduced in comparison to the replication of H5N1 HK03 ca or H1N1 NC99 ca viruses. The VN04 ca virus is attenuated in replication in the lower respiratory tract, and it replicates in the upper respiratory tract of ferrets at a level much lower than does the HK03 ca virus, presumably due to its avian receptor-binding preference and glycosylation at the 158N site (Wang et al., 2010). In addition, it is possible that the higher cytokine induction by the VN04 ca virus infection might also contribute to the poor replication of the VN04 ca virus in the respiratory tract of ferrets and result in a poor antibody response. A human study indicated that increased production of IP-10 (CXCL10) in elderly correlates with a low antibody response to the seasonal inactivated influenza vaccine (Corsini et al., 2006), suggesting the necessity of evaluating cytokine gene expression in vaccine-virus–vaccinated subjects. LAIV-vaccinated children had a greater overexpression of genes involved in induction and activation of IFN related pathway; therefore, the transcriptional response to influenza vaccine may correlate with vaccine-induced adaptive response (Zhu et al., 2010). More studies are needed to understand the impact of cytokine induction in relation to viral pathogenesis and vaccine virus replication in the host.

Materials and methods

Virus

Live attenuated H5N1 influenza virus vaccines containing the NA gene and the cleavage site-modified HA gene from viruses isolated from humans in 2003 and 2004 (A/Hong Kong/213/03 and A/Vietnam/1203/04) were described previously (Suguitan et al., 2006). To introduce specific mutations into the HA gene, site-directed mutagenesis of HA plasmid was performed. Influenza A/New Caledonia/20/99 ca (H1N1) (NC99 ca) vaccine virus, and the reassortant H5N1 ca viruses containing the HA and NA gene segments of either the H5N1 HK03 ca, VN04 ca, H1N1 NC99 ca, or H2N2 AA60 ca virus were generated by plasmid rescue as previously described (Jin et al., 2003). The reassortant viruses were designated VN04-HA/HK03-NA ca, VN04-HA/NC99-NA ca, or HK03-HA/VN04-NA ca. To introduce specific mutations into the HA gene, site-directed mutagenesis of HA plasmid was performed, and reassortant viruses were rescued by plasmid rescue. Viruses were amplified in the allantoic cavity of 10–11-day-old embryonated SPFAS hen’s eggs (Charles River, Norwich, CT) at 33°C for 3 days, and their HA and NA gene segments were confirmed by sequencing. Allantoic fluids collected from the infected eggs were quantitated by HA assay using 0.5% turkey or horse erythrocytes in PBS or by plaque assay using Madin–Darby canine kidney (MDCK) cells (American Type Culture
Collection, ATCC, Manassas, VA), β-Propiolactone- (BPL; Sigma, St. Louis, MO) inactivated viruses were prepared by incubating viruses with 0.5% BPL in PBS at 37 °C for 2 hr, followed by overnight dialysis at 4 °C in phosphate-buffered solution (PBS, pH 7.0 to pH 7.6). Complete virus inactivation was confirmed by plaque assay in MDCK cells.

**Viral infection and plaque assay**

A549 cells (carcinomic alveolar basal epithelia cell line; CCL-185) from ATCC were cultured in Eagle's minimal essential medium (Sigma, St. Louis, MO) with 10% fetal bovine serum and seeded onto 6-well plates at 5 x 10⁵ cells per well. Cell monolayers were washed with PBS and infected with virus. After absorption at room temperature for 1 hr, the cell monolayers were washed with PBS and incubated with OPTI-MEM I medium (Invitrogen, Carlsbad, CA). Culture supernatants were collected at 6, 24, and 48 hr post-infection (PI) to examine virus titer and levels of cytokines. One set of the infected cells was collected at 6 hr PI and at 24 hr PI (each) for RNA extraction to examine levels of cytokine gene expression, whereas other sets of the infected cells were examined for virus antigens by immunofluorescence staining or by Western blotting. Plaque assay was performed in MDCK cells, and plaques were enumerated by immunostaining as previously described (Jin et al., 2003).

**Immunofluorescence staining**

Influenza virus-infected cells were fixed in 3.7% formaldehyde at room temperature for 15 min and then penetrated with cold 100% methanol for 5 min at −20 °C. The cells were incubated with mouse anti-influenza A nucleocapsid protein (NP) monoclonal antibody (MAB8252, Chemicon, Temecula, CA), followed by staining with fluorescent isothiocyanate-conjugated anti-mouse antibody (Invitrogen) and counterstained with Hoechst stain (Invitrogen) to visualize nuclei.

**Western blotting**

Virus-infected A549 cells were collected at 6 hr PI in Laemml sample buffer (Bio-Rad, Hercules, CA) and electrophoresed on 4–20% Tris–glycine precast gel (Invitrogen). The proteins were transferred to a PVDF membrane (Invitrogen) and incubated with chicken polyclonal antibody against A/Ann Arbor/6/60 followed by horse radish peroxidase-conjugated goat anti-chicken antibody. The protein–antibody complexes on the membrane were detected on X-ray film following incubation with enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

**cDNA microarray analysis**

Total cellular RNAs were isolated from virus-infected cells using RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with DNase I. The quantity of RNA was assessed by an ultraviolet spectrophotometer and the quality of RNA was analyzed by 2100 Bioanalyzer (Agilent, Foster City, CA). Total RNA (4 μg) was used as template to synthesize single-stranded cDNA using Superscript II reverse transcriptase and an T7-oligo (dT) primer followed by synthesis of the complementary strand cDNA using a combination of DNA polymerase I, Escherichia coli DNA ligase, and RNase H. The cDNA was labeled with biotinylated ribonucleotide using T7 RNA polymerase and fragmented by metal-induced hydrolysis. An equal amount of cDNA (15 μg) of each sample was hybridized to an Affymetrix human U133A plus 2.0 GeneChip array and detected with streptavidin phycoerythrin. Each array was scanned, inspected, and analyzed according to the manufacturer’s instructions. The data were transferred to GeneSpring GX software, version 10 (Agilent Technologies, Inc, Santa Clara, CA) for annotation, normalization, filtering, and integration. The GC Robust Multiaarray Averaging algorithm was used to normalize the data followed by baseline transformation. For the comparison of gene expression profiles between infected and uninfected samples, a filter on a volcano plot of treatment versus control program was applied. A standard Student’s t test was performed between the virus-infected and uninfected groups. Genes expressed at levels of 2-fold or higher in the infected samples compared to the uninfected samples, with P value <0.05, were identified for in-depth comparisons.

**Quantitative reverse transcription–PCR (qRT–PCR)**

A subset of the genes that were identified to be upregulated in the infected cells by microarray analysis was further examined by qRT–PCR analysis. The primers used for qRT–PCR of IL-6, IFN-β, RIG-I, and ISG-15 were reported previously (Blaschke et al., 2000; Zeng et al., 2007). Reverse transcription was performed using Superscript III reverse transcriptase and Oligo-dT primers (Qiagen). Quantitative RT–PCR reactions were set up in triplicate using a SYBR green master mix and Stratagene MX3005P PCR system (Agilent Technologies). Levels of gene expression for each sample were normalized to that of glyceraldehyde-3-phosphate dehydrogenase in the same sample.

**Enzyme-linked immunosorbent assay (ELISA)**

Levels of IL-6, IL-8, CXCL10 (IP10), CCL5 (RANTES), and CCL2 (MCP-1) proteins in virus-infected A549 cell culture supernatants were measured using gene-specific ELISA kits (R&D Systems). The live viruses present in the culture supernatants were inactivated by irradiation in Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) for 2 min prior to performing the ELISA assay.

**Examination of the interferon (IFN) promoter activity**

The p125Luc reporter plasmid containing the firefly luciferase gene (FF-Luc) under the control of IFN-β promoter (Yoneyama and Fujita, 2007) was kindly provided by Dr. Takashi Fujita (Kyoto, Japan). The pGL-4 promisc (Promega, Madison, WI) encoding the Renilla luciferase gene (REN-Luc) was used as the internal transfection control. A549 cells were cotransfected with 1 μg of the FF-Luc and 0.1 μg of the REN-Luc plasmid using Lipofectamine 2000 (Invitrogen). At 16 hr post-transfection, the cells were superinfected with influenza viruses at an MOI of 3.0 for 24 hr, and the cells were lysed in 100 μl lysis buffer (Promega). An aliquot of 20 μl of cell lysate was assessed using Dual-Glo Luciferase Reporter Assay System (Promega). The luminescent signal was measured using GloMax 96-well Microplate Luminometer (Promega). The FF-Luc activity was normalized to the REN-Luc activity and expressed as the IFN-β promoter activity. Statistical analysis was performed using Student’s t-test, and the differences were considered significant at P<0.05.

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